JOURNAL OF BACTERIOLOGY, June 1994, p. 3460–3465
0021-9193/94/$04.00+0

Vol. 176, No. 12

Purification and In Vitro Activities of the Native Nitrogen Fixation Control Proteins NifA and NifL

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Received 24 January 1994/Accepted 5 April 1994

The prokaryotic enhancer-binding protein NifA stimulates transcription at a distance by binding to sequences upstream of nitrogen fixation (*nif*) promoters and catalyzing the formation of open promoter complexes by RNA polymerase containing the alternative sigma factor, σ^{54}. The activity of NifA in vivo is modulated by the negative regulatory protein NifL in response to environmental oxygen and fixed nitrogen. To date, a detailed biochemical analysis of these proteins from the model diazotroph *Klebsiella pneumoniae* has been hindered by their insolubility. We have now purified NifA and NifL from *Azotobacter vinelandii* in their native form. NifA is competent in specific DNA binding, transcriptional activation, and response to negative regulation by NifL in vitro. In contrast to the conserved mechanism of phosphotransfer demonstrated by other two-component regulatory systems, our results support a model in which NifL regulates the activity of NifA via a protein-protein steric block interaction rather than a catalytic modification of NifA.

Formation of productive open complexes by the alternative RNA polymerase holoenzyme containing σ^{54} requires an activator protein, the activity and synthesis of which are regulated in response to environmental effectors (16). In many diazotrophs, activation of nitrogen fixation genes requires the NifA activator protein, a DNA-binding protein which recognizes upstream promoter sequences showing many properties of enhancer elements (5). *Azotobacter vinelandii* synthesizes three distinct nitrogenases. Each system has its own set of nitrogenase structural genes, and a specific σ^{54}-dependent activator protein is required for the expression of these genes: NifA for *nifHDK*, VnfA for *vnfHDGK*, and AnfA for *anfHDGK* (15). The integration host factor protein (IHF) stimulates NifA-mediated activation of *nif* promoters by facilitating DNA loop formation and productive interactions between $E\sigma^{54}$ and NifA (14, 22). A binding site for IHF is present between the $E\sigma^{54}$ binding site and the NifA upstream activator sequence (UAS).

A. vinelandii NifA has the three-domain structure characteristic of the family of σ^{54}-dependent activator proteins (10; reviewed in reference 20). The N-terminal domain shows the least homology to the *Klebsiella pneumoniae* protein, but the central domain and C-terminal DNA-binding domain are highly conserved between the two proteins. The sequence of the putative DNA recognition helix in *A. vinelandii* NifA is homologous to that conserved in other NifA proteins and reflects a common DNA binding site (UAS). In *K. pneumoniae*, the activity of the NifA protein is inhibited by the NifL protein in response to fixed nitrogen and oxygen by a mechanism which probably involves formation of a NifA-NifL complex (13). A *nifL* gene has recently been identified and sequenced in *A. vinelandii* (2, 21). However, compared with the *K. pneumoniae* NifL sequence, the predicted amino acid sequence of the *A. vinelandii* protein shows greater homology to the histidine protein kinase family of bacterial two-component systems. In particular, the conserved histidine residue shown to be the site of autophosphorylation in other members of this family is present in the C-terminal domain, whereas this histidine residue is not present in the *K. pneumoniae* NifL protein. It has been suggested that the mechanism of inhibition of NifA by NifL in *A. vinelandii* may involve phosphorylation or phosphotransfer (reviewed in reference 3).

NifA activity, the involvement of IHF, and inhibition of NifA by NifL have been demonstrated in coupled transcription-translation systems, but further attempts to purify the NifA protein have been thwarted by loss of activity and aggregation during chromatography (1, 3, 22). Recently, the *K. pneumoniae* NifA protein has been purified as a fusion protein with the maltose-binding protein (MBP) (17). The fusion protein (MBP-NifA) has activity in vitro but is still aggregated, and release of MBP results in loss of activity and insolubility of the NifA protein. We have now shown that the wild-type *A. vinelandii* NifA and NifL can be purified in soluble, active forms, and this has enabled us to demonstrate the regulatory activity of these native proteins in defined systems in vitro.

MATERIALS AND METHODS

Purification of NifA from *A. vinelandii*. Overproduction was achieved by using the T7 expression system. Synthesis of NifA was induced in 1- to 2-liter cultures of *Escherichia coli* BL21(DE3) (23) carrying plasmid pDB737. This plasmid contains the *nifA* gene cloned into the expression vector pT7-7. PCR-generated restriction sites, *Nde*I for the *nifA* start codon and *Bam*HI downstream of the stop codon, were used to clone the gene into the expression vector. The complete nucleotide sequence of the PCR product confirmed that the coding sequence was identical to that of the wild-type gene. Induction of aerobically grown cultures with 0.5 mM isopropylthiogalactopyranoside (IPTG) for 3 to 4 h at 28°C in Luria broth resulted in the synthesis of high levels of NifA protein. Crude cell extract was obtained by French pressure disruption and low-speed centrifugation of the resulting lysate. The supernatant contained at least 50% of the overproduced protein; the rest sedimented with the cell debris. NifA was precipitated with ammonium sulfate to 55% saturation. The precipitate was dissolved in TGED buffer (10 mM Tris-Cl [pH 8], 5% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol) containing 50 mM NaCl and chromatographed on heparin-agarose with a linear gradi-

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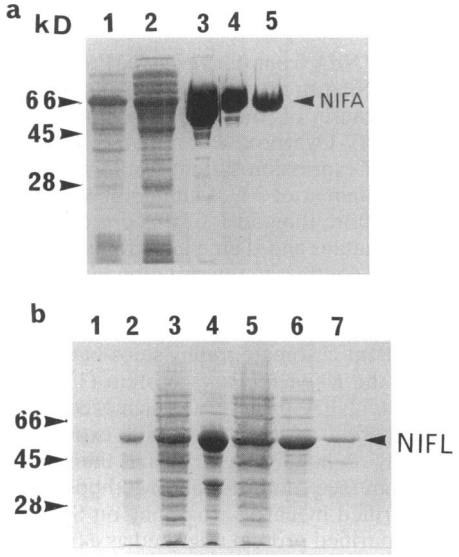

FIG. 1. (a) Purification of *A. vinelandii* NifA. Various stages in the purification of NIFA are shown. Lanes: 1, whole cell extract containing NifA overexpressed after IPTG induction; 2, crude cell extract supernatant; 3, heparin-agarose fraction; 4, Mono Q fraction; 5, Superose 12 fraction. (b) Purification of *A. vinelandii* NifL. Various stages in the purification of NIFL are shown. Lanes: 1 and 2, whole cell extract from cells containing the NifL overexpression plasmid before (lane 1) and 3 h after (lane 2) induction with IPTG; 3, crude cell extract supernatant; 4, low-speed pellet from cell extract; 5, ammonium sulfate fraction; 6, Econopac Q fraction; 7, phenyl-Superose fraction.

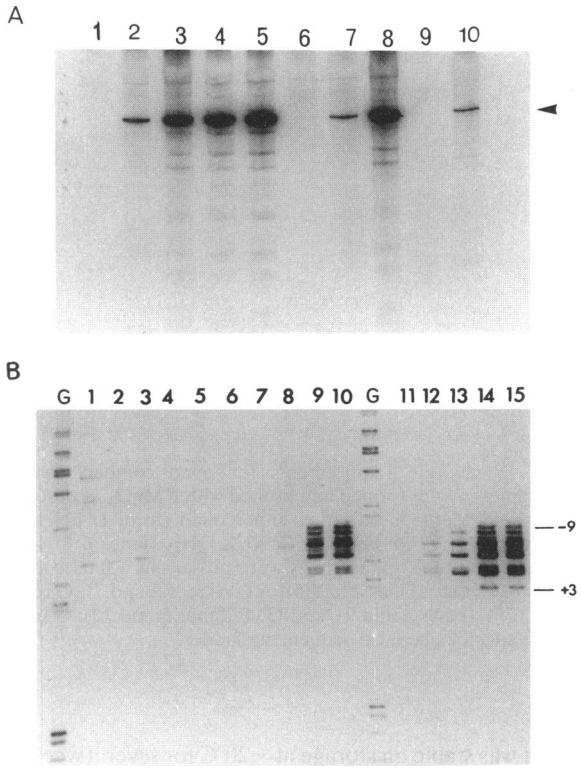

FIG. 2. (A) Transcriptional activation by NifA. Single-round transcription assays were carried out in TAP buffer. Template DNA (10 nM) was preincubated at 30°C for 20 min with core RNA polymerase (75 nM), σ^{54} (230 nM), ATP, GTP, and CTP (0.4 mM each), and NifA (25 or 250 nM) to allow accumulation of open complexes. When present, IHF was used at a final concentration of 50 nM. A mix containing heparin (100 μg/ml, final concentration) and 12.5 μM [α-^{32}P]UTP was then added, and incubation continued for 10 min to allow synthesis of transcripts. Reaction products were phenol extracted, ethanol precipitated, and then run on polyacrylamide sequencing gels; radioactive bands were identified following autoradiography, and those corresponding to full-length transcripts were excised from the gels and scintillation counted in Cocktail T (BDH). In lanes 1 to 5, template DNA was pNH8 containing the *nifH* promoter and upstream binding sites for NifA and IHF but lacking the *nifJ* UAS; in lanes 6 to 10, template DNA was pNH7, which lacks the *nifH* UAS. Lanes 1 and 6 are controls without NifA. In lanes 2, 4, 7, and 9, the NifA concentration was 25 nM; in lanes 3, 5, 8, and 10, the NifA concentration was 250 nM. In lanes 4, 5, 9, and 10, 50 nM IHF was present. The counts per minute after subtracting the background values in lanes 1 and 6 were as follows: lane 2, 6,672; lane 3, 30,851; lane 4, 35,846; lane 5, 55,325; lane 7, 3,501; lane 8, 42,413; lane 9, 228; and lane 10, 3,036. (B) Potassium permanganate footprinting was carried out as described in Materials and Methods, using the following DNA templates; *Pst*I-digested linear DNA from pNH7 (lacking the UAS; lanes 1 to 5), *Pst*I-digested linear DNA from pNH8 (lanes 6 to 10), or form I supercoiled pNH8 DNA (lanes 11 to 15). ATP (final concentration, 4 mM) was present in all reaction mixtures. The NifA concentration was either 25 nM (lanes 2, 4, 7, 9, 12, and 14) or 200 nM (lanes 3, 5, 8, 10, 13, and 15). IHF (50 nM) was present in lanes 4, 5, 9, 10, 14, and 15. G indicates a guanine-specific chemical sequencing ladder.

ent from 50 to 500 mM NaCl. NifA eluted at 0.36 M NaCl. The peak fractions were pooled and applied to a 1-ml Mono Q column, where the protein eluted at 0.3 M NaCl. Further purification was achieved by gel filtration on Superose 12. NifA eluted from this column as a broad peak, and the fractions were analyzed by native gel electrophoresis and Western blotting (immunoblotting) (see Results). *A. vinelandii* NifA cross-reacted with a polyclonal rabbit antiserum raised to the NifA protein from *K. pneumoniae*. NifA was stored in TGED buffer containing 50 mM NaCl and 50% glycerol in liquid N_2, where it was stable for several months. NifA was unstable at −20°C and lost activity over several days. The reason for the instability was apparently not due to proteolytic degradation, as the correct size protein was still detected on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The identity of the protein was confirmed by N-terminal sequence analysis of the first nine residues of the purified protein. The concentration of NifA was calculated by assuming the protein to be a dimer.

Purification of NifL from *A. vinelandii*. An overproducing plasmid, pPW53, was constructed by inserting the *nifL* coding sequence into pT7-7. PCR-generated restriction sites, *Nde*I for the *nifL* start codon and *Eco*RI to overlap the stop codon, were used to clone the gene. Sequencing of the PCR product confirmed that this directed the synthesis of the wild-type protein. The NifL protein was induced as for NifA. On French pressure disruption and low-speed centrifugation of the crude cell lysate, approximately 75% of the NifL sedimented with the cell debris. Purification of NifL from the supernatant was achieved by ammonium sulfate fractionation to 55% saturation, followed by chromatography in TGED buffer on an Econopac Q column (Bio-Rad), where the protein bound weakly and eluted at 200 mM NaCl. The peak fractions were pooled and dialyzed against 1 M ammonium sulfate and applied to a 1-ml phenyl-Superose column on the fast protein liquid chromatograph (FPLC). The column was developed with a descending salt gradient from 1 M ammonium sulfate, and the protein eluted in TGED buffer without ammonium sulfate. The purified NifL was stored in the same buffer as

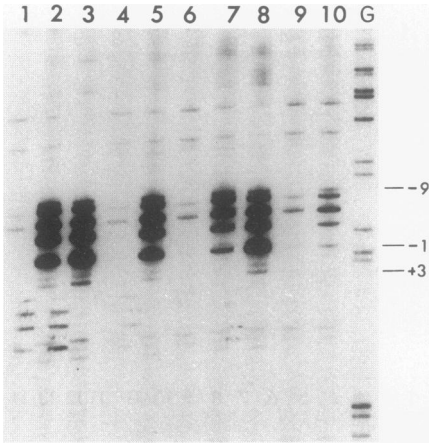

FIG. 3. Nucleotide requirements for open complex formation. Open complexes were formed and probed with $KMnO_4$ as described in Materials and Methods on either supercoiled (form I) pNH8 DNA (lanes 1 to 5) or *Eco*RI-linearized pNH8 DNA (lanes 6 to 10). The following nucleoside triphosphates were present at a final concentration of 1 mM: none (lanes 1 and 6), ATP (lanes 2 and 7), GTP (lanes 3 and 8), CTP (lanes 4 and 9), and UTP (lanes 5 and 10). G indicates a guanine-specific chemical sequencing ladder.

NifA but was stable on storage at −20°C for several weeks. The concentration of NifL was calculated by assuming the protein to be a dimer, and the identity was confirmed by N-terminal sequence analysis of the first nine residues of the purified protein.

RNA polymerase and σ^{54} from *K. pneumoniae* were purified as described previously (25). *E. coli* IHF protein was the kind gift of Howard Nash.

Transcription assays. Single-round transcription assays with purified proteins were carried out as described previously (9). All reactions were carried out in TAP buffer (9) in a final volume of 50 μl. Template DNAs (10 nM) were pNH8, which carries the *nifH* promoter fragment from pSMM8 in the transcription vector pTE103, and pNH7, which contains the *nifH* promoter fragment lacking the NifA UAS from pSMM4 in pTE103 (5, 12). Core RNA polymerase, σ^{54}, IHF, and NifA were used at the concentrations indicated in the figure legends. ATP, GTP, and CTP (0.4 mM) were added prior to the heparin challenge to allow formation of initiated open complexes. Open complexes were stable for more than 20 min on supercoiled DNA when only GTP (1 mM) was present.

DNA binding. Binding of NifA to promoter sequences was assayed in vitro at 30°C essentially as previously described (4) on linear DNA templates with DNase I and dimethyl sulfate. For these experiments, TAP buffer was used. *ortho*-Copper phenanthroline footprinting was conducted in STA buffer supplemented with 80 mM KCl but without dithiothreitol in the 5× stock buffer (20a).

Open complex formation. Potassium permanganate footprinting was carried out essentially as described previously (25). Open complexes were formed by incubating 5 nM template DNA with 75 nM core RNA polymerase, 220 nM σ^{54}, NifA, and an appropriate nucleoside triphosphate (at concentrations indicated in the figure legends) for 20 min at 30°C. IHF (when present) was added at a final concentration of 50 nM. Open complex formation was probed for by adding $KMnO_4$ (final concentration, 8 mM), which was quenched after 4 min of incubation.

RESULTS

Purification of NifA from *A. vinelandii*. Previous attempts to purify wild-type NifA protein from other organisms have been unsuccessful because of the very insoluble nature of the protein (1, 22, 24). Overproduction of the *A. vinelandii* NifA protein in the T7 expression system results in the synthesis of high levels of protein after 3 h of induction with IPTG (Fig. 1). Upon cell lysis, more than 50% of this protein remains in the low-speed supernatant and is amenable to purification (Fig. 1). This is in marked contrast to the situation in *K. pneumoniae*, in which case only a very low level of the overproduced NifA remains in the cell supernatant. Loss of activity and aggregation on subsequent chromatography steps have precluded any purification of the *K. pneumoniae* protein (1). Purification of the *A. vinelandii* NifA protein was achieved by ammonium sulfate fractionation of the crude cell extract followed by chromatography on heparin-agarose and then on Mono Q on the FPLC system (see Materials and Methods). Some protein was further purified by chromatography on Superose 12, also by FPLC. The purified protein migrated as a band corresponding to a subunit molecular mass of 66 kDa on SDS-PAGE. However on native gel electrophoresis, the protein migrated as a range of species from approximately 120-kDa to higher-molecular-mass aggregates. On gel filtration on Superose 12, the NifA eluted as a broad peak. Analysis of these fractions by native gel electrophoresis and Western blotting revealed that they also contained a range of species from the dimer form upwards but that the population of higher-molecular-weight material was absent.

Transcriptional activation. Two aspects of positive control by purified NifA were examined in vitro: dependence on the presence of the UAS and the requirement for IHF for stimulation of transcriptional activation. In the absence of IHF, NifA activated transcription by $E\sigma^{54}$ from the *nifH* promoter on supercoiled DNA templates whether or not the *nifH* UAS was present (Fig. 2A; compare lanes 2 and 3 with lanes 7 and 8). The ability of NifA to promote UAS-independent activation is apparently sensitive to the topological nature of the template DNA, since a significant level of open complex formation was not observed on linear DNA templates in the absence of IHF (Fig. 2B; compare lanes 2 and 3 and lanes 7 and 8). IHF stimulated the formation of open promoter complexes and specific transcripts on supercoiled DNA when the *nifH* UAS was present (Fig. 2A, lanes 2 to 5; Fig. 2B, lanes 12 to 15). At subsaturating concentrations of NifA (25 nM), the level of stimulation by IHF was 5-fold as measured by the transcription assay and 10-fold with respect to open complex formation.

The requirement for IHF was particularly stringent on linear DNA templates (Fig. 2B, lanes 1 to 10). Provided that the UAS was present, stimulation of open complex formation by IHF was 340-fold on linear DNA at 25 nM NifA and 28-fold when the NifA concentration was saturating (200 nM). No IHF-dependent stimulation was observed in the absence of the UAS; in fact, IHF inhibited transcriptional activation by NifA on a supercoiled DNA template lacking the UAS but containing the IHF binding site (Fig. 2A, lanes 6 to 10). Presumably under these conditions, transcriptional activation by NifA requires direct contact between the activator and $E\sigma^{54}$, perhaps potentiated by nonspecific DNA binding. One interpretation of this result is that bound IHF occludes formation of the complex between NifA and $E\sigma^{54}$.

In marked contrast to NifA from *K. pneumoniae* (1, 22), the activity of *A. vinelandii* NifA was not apparently temperature sensitive in vitro, since transcriptional activation was not

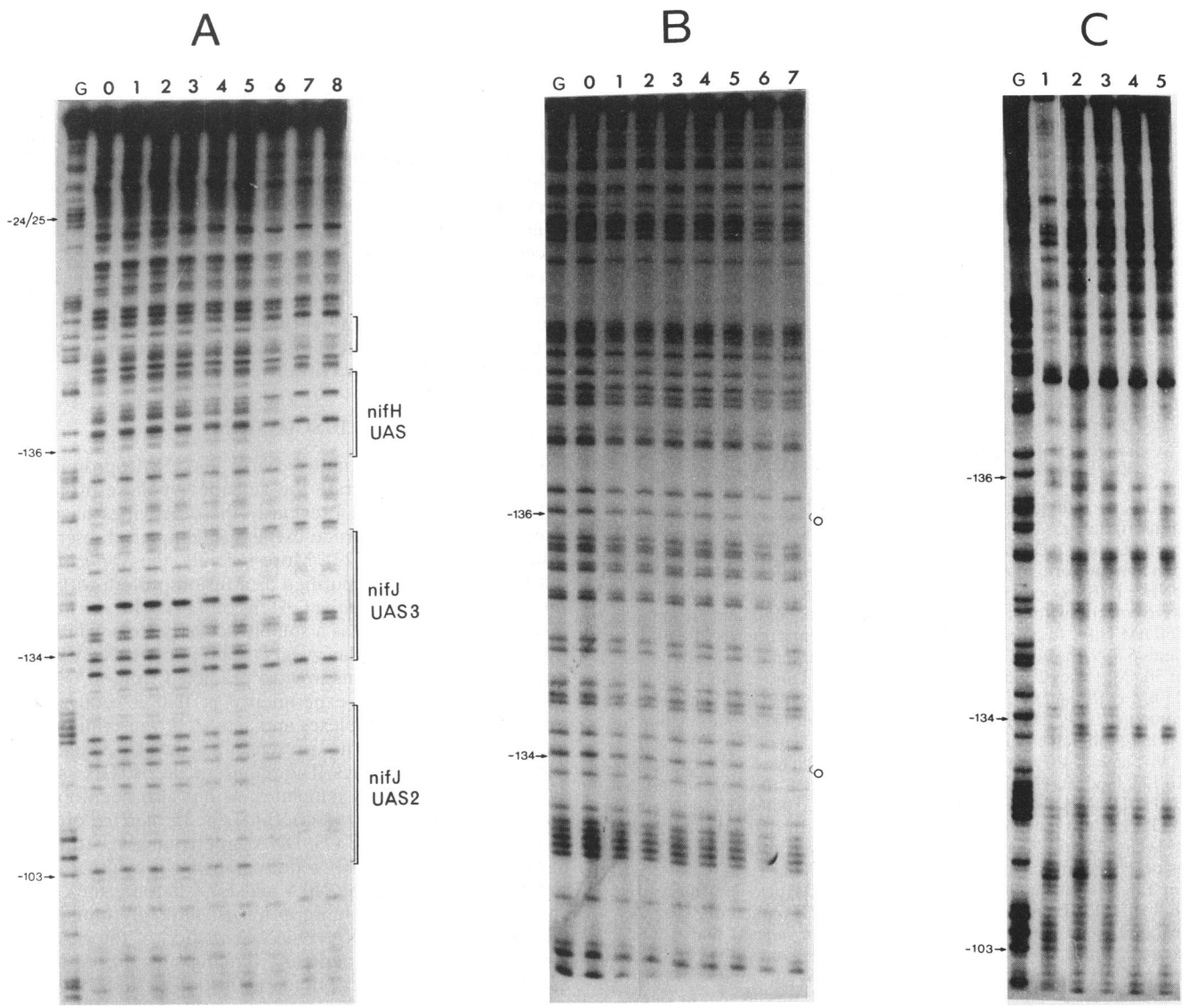

FIG. 4. Specific binding of NifA to the *K. pneumoniae nifH* and *nifJ* promoter regions. Panels A, B, and C are DNase I, dimethyl sulfate, and *ortho*-copper phenanthroline footprints, respectively. Lane G is chemical guanine sequencing ladder; lane 0 contains no NifA. For panel A, NifA was present at 25 nM (lane 1), 50 nM (lane 2), 100 nM (lane 3), 200 nM (lane 4), 400 nM (lane 5), 800 nM (lane 6), 1.6 μM (lane 7), and 3.2 μM (lane 8). NifA concentrations in panel B were as follows: lane 1, 25 nM; lane 2, 50 nM; lane 3, 100 nM; lane 4, 200 nM; lane 5, 400 nM; lane 6, 800 nM; and lane 7, 1.6 μM. (C) Lane 1, no NifA; lane 2, 200 nM NifA; lane 3, 400 nM NifA; lane 4, 800 nM NifA; lane 5, 1.6 μM NifA. Guanine residues of the *nifH* and *nifJ* UASs are indicated, numbered relative to their transcription starts. The binding activity of a number of independent NifA preparations was assayed and found to be very comparable to that depicted. However, fractionation of NifA upon Superose 12 chromatography generated fractions with three to five times this binding activity. This increase in binding activity did not correlate simply with size distribution of the fractions, indicating that an increase in abundance of the dimer-tetramer form was not solely responsible for the increased activity.

significantly decreased in assays at 37°C compared with 30°C. Preincubation of *A. vinelandii* NifA for 20 min at 37°C also did not influence activity (data not shown).

Nucleoside triphosphate requirements for open complex formation. In common with NtrC and NifA from *K. pneumoniae*, open complex formation by *A. vinelandii* NifA required a nucleoside triphosphate. On a supercoiled DNA template in the presence of $E\sigma^{54}$ and IHF, *A. vinelandii* NifA could use ATP, GTP, or UTP, but not CTP, in contrast to the *K. pneumoniae* MBP-NifA (17), to promote open complexes in which pyrimidine residues from −9 to +3 are reactive to potassium permanganate (Fig. 3, lanes 1 to 5). Similar results were obtained on a linear DNA template with the exception that UTP appeared to be relatively inefficient (Fig. 3, lanes 6 to 10). We assume that nucleotide hydrolysis is required for the catalysis of open promoter complexes, since neither ATPγS nor ADP could support their formation (data not shown).

Specific binding of NifA to UASs. Purified *A. vinelandii* NifA bound to three sites in the upstream sequences of the *K. pneumoniae nifH* promoter previously identified by mutagenesis and in vivo footprinting to be NifA binding sites (6, 18). These were the *nifH* UAS and the two *nifJ* UASs. Binding was

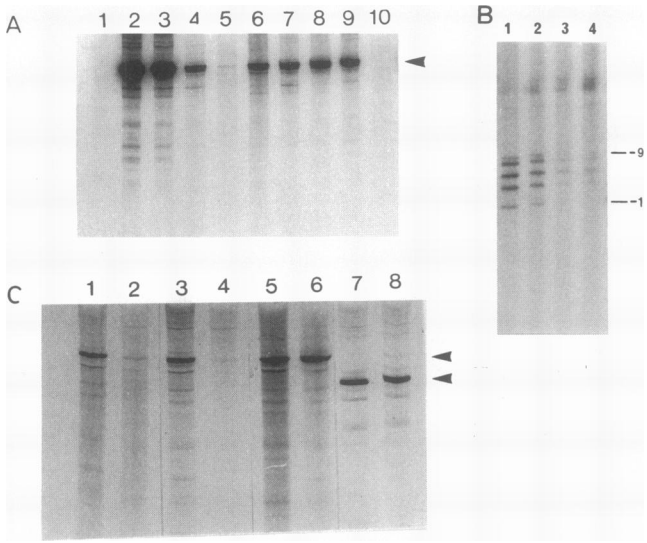

FIG. 5. Inhibition of NifA activity by NifL. (A) Single-round transcription assays were carried out as described for Fig. 2A. All proteins were incubated together with template DNA at 30°C for 20 min to allow formation of open complexes. Template DNA was 10 nM pNH8. IHF was used at a final concentration of 50 nM. Lanes 1 and 10 were controls without activator protein; lane 10 contained, in addition, 250 nM NifL. In lanes 2 to 5, the NifA concentration was 100 nM. In lanes 6 to 9, the NtrC concentration was 250 nM and NtrB was present at 30 nM. Lanes 2 and 6 did not contain NifL. Lanes 3 and 7 contained 25 nM NifL, lanes 4 and 8 contained 100 nM NifL, and lanes 5 and 9 contained 250 nM NifL. Counts per minute after subtraction of background values in lane 1: lane 2, 90,898; lane 3, 65,235; lane 4, 9,861; lane 5, 467; lane 6, 12,841; lane 7, 13,925; lane 8, 15,053; lane 9, 16,125; lane 10, 471. (B) Open complexes were formed on supercoiled pNH8 DNA in the presence of 200 nM NifA, 50 nM IHF, and 4 mM ATP. NifL was not added (lane 1) or was added at 100 nM (lane 2), 200 nM (lane 3), and 300 nM (lane 4). Reactions were probed with $KMnO_4$ as described in Materials and Methods. (C) Assay conditions were as for Fig. 2A except that [^{35}S]UTPαS (12.5 μM) was used instead of [α-^{32}P]UTP. Template DNA (10 nM) was pNH8 (containing the UAS) in lanes 1, 2, 5, and 6. pNH7 (no UAS) in lanes 3 and 4. pSA8 containing the *anfH* promoter in lanes 7 and 8. Lanes 1 to 4 contained 100 nM NIFA. Lanes 5 and 6 contained 250 nM NtrC and 30 nM NtrB; lanes 7 and 8 contained 100 nM ΔAnfA. NifL was present in lanes 2, 4, 6, and 8 at 170 nM.

detected by methylation protection of the guanine residues of the TGT motif of the UAS, DNase I protection, and *ortho*-copper phenanthroline footprinting (Fig. 4). In the DNase I footprints, some weak protection of a site downstream of the NifH UAS was evident (bracketed in Fig. 4A). This is located between −107 and −92, and the presence of TGT motifs at −106 and −96 may indicate weak half sites. As judged by the ability of *ortho*-copper phenanthroline to detect conformational change in DNA, binding of NifA alone to DNA does not grossly change its conformation. Footprints of NifA on the other DNA strand confirmed the conclusions drawn from Fig. 4 (data not shown).

Specific inhibition of NifA activity by NifL. We overproduced the NifL protein aerobically in rich medium in *E. coli*, ensuring that it was in its repressive form (13). The effect of purified *A. vinelandii* NifL protein on the formation of transcriptionally competent initiation complexes by NifA was examined in the transcription assay. NifA-mediated activation was partially inhibited when an equimolar concentration of NifL was added and completely inhibited when NifL was in excess (Fig. 5A, lanes 1 to 5), whereas NifL had no effect on activation by phosphorylated NtrC under these conditions (Fig. 5A and C). The specificity of inactivation was further demonstrated by the observation that NifL did not influence activation of the *anfH* promoter by an N-terminally truncated form of AnfA (ΔAnfA), an Eσ^{54}-dependent activator required for expression of the third nitrogenase system in *A. vinelandii* (Fig. 5C, lanes 7 and 8). Stoichiometric concentrations of NifL prevented the formation of open promoter complexes by NifA (Fig. 5B). Inactivation of NifA by NifL is not reliant on the specific DNA binding function of NifA, since inactivation occurred in the absence of the UAS (Fig. 5C, lanes 3 and 4). The requirement for stoichiometric amounts of NifL to inhibit the activity of NifA suggests that inactivation of NifA is likely to occur by protein-protein interaction rather than by catalytic modification of NifA by NifL.

DISCUSSION

Purification of the native NifA and NifL proteins has enabled us to demonstrate the activities of these proteins in defined in vitro assays. Previously these activities have been demonstrated only in crude extracts in coupled transcription-translation systems (1, 22). More recently, the *K. pneumoniae* NifA protein has been purified as a fusion protein with MPB, providing a large N-terminal extension (17). The *A. vinelandii* protein can be purified as a soluble active protein exhibiting a range of native molecular weights. The purified NifA showed positive control functions in vitro predicted from in vivo (19) and other (see above) experiments, in that it catalyzed the formation of open complexes and activated transcription from the *nifH* promoter. The presence of the UAS and IHF stimulated these activities. The requirement for IHF was most evident on linear DNA templates, as has also been observed for IHF stimulation of transcription from the *glnHp2* promoter by phosphorylated NtrC (7). DNA supercoiling may therefore partially replace the IHF requirement by providing a more suitable alignment of the NifA and Eσ^{54} binding sites. In the absence of the UAS, transcription is markedly inhibited by IHF on supercoiled DNA. One explanation in the case cited above is that the NifA and Eσ^{54} binding sites are misaligned and IHF inhibits transcriptional activation by inducing DNA bending in an unfavorable orientation (8). Alternatively, IHF may directly interfere with the NifA interaction with Eσ^{54} at the promoter. Although we find that NifA binds specifically to UASs, the concentrations of NifA required to detect UAS occupancy by footprinting were significantly higher than those which were saturating with respect to open complex formation (200 nM). Whether this reflects cooperative interactions between NifA and Eσ^{54} during transcription initiation or dissociation of NifA from the template DNA and activation of other templates during the time course of the assay is not distinguished by our experiments. Unexpectedly, the activity of the NifA protein was not significantly reduced by treatment at 37°C. The relative thermal stability of *A. vinelandii* NifA may indicate that the protein has a more stably folded conformation than the *K. pneumoniae* protein, which may influence both its solubility and its narrower nucleotide specificity compared with the *K. pneumoniae* MBP fusion protein (17).

We have shown that addition of stoichiometric amounts of NifL inhibits formation of open complexes. This is consistent with previous results from immunoprecipitation experiments using *K. pneumoniae* proteins in cell extracts. Antisera against either NifA or NifL precipitated both proteins, indicating that they form a complex in vivo and that the modulation of NifA activity by NifL may occur via a protein-protein interaction

(13). Preliminary evidence suggests that NifL does not markedly inhibit DNA binding by NifA in vitro (unpublished observation) and that an inhibitory effect of NifL on NifA occurs whether or not the UAS is present in the promoter DNA. Therefore, it seems likely that one target for NifL is the positive control function of NifA. Thus, NifL could inhibit a NifA-Eσ^{54} interaction required for open complex formation, possibly by inhibiting the ATPase activity of NifA. Since preliminary evidence suggests that phosphotransfer from NifL to NifA does not appear to be involved, at least under the conditions tested (unpublished observation), the interaction of NifL with NifA may have evolved from the standard two-component systems by transforming protein-protein interaction into a steric block, making phosphorylation no longer a requirement (3). It seems unlikely that a catalytic modification of the N-terminal domain NifA is taking place, and it may be that NifL interacts directly with the central domain of NifA to block its activity. This is consistent with evidence from *K. pneumoniae*, in which case removal of the N-terminal domain of NifA makes it particularly sensitive to inhibition by NifL in vivo. One role of the N-terminal domain of NIFA may be to block the inhibitory effect of NifL on NifA positive control function (11).

ACKNOWLEDGMENTS

We thank Dennis Dean and Valerie Cash for providing plasmid pDB737 and for initial observations on the nature of the overproduced NifA. We are also grateful to Martin Drummond and Paul Woodley for providing plasmid pPW53. We thank Martin Drummond, Mike Merrick, and Barry Smith for comments on the manuscript and Carol Sterenberg for typing it.

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